Video Article

Protocol for Three-dimensional Confocal Morphometric Analysis of Astrocytes

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Abstract

As glial cells in the brain, astrocytes have diverse functional roles in the central nervous system. In the presence of harmful stimuli, astrocytes modify their functional and structural properties, a condition called reactive astrogliosis. Here, a protocol for assessment of the morphological properties of astrocytes is presented. This protocol includes quantification of 12 different parameters *i.e.* the surface area and volume of the tissue covered by an astrocyte (astrocyte territory), the entire astrocyte including branches, cell body, and nucleus, as well as total length and number of branches, the intensity of fluorescence immunoreactivity of antibodies used for astrocyte detection, and astrocyte density (number/1,000 μm^2). For this purpose three-dimensional (3D) confocal microscopic images were created, and 3D image analysis software such as Volocity 6.3 was used for measurements. Rat brain tissue exposed to amyloid beta$_{1-40}$ ($A\beta_{1-40}$) with or without a therapeutic intervention was used to present the method. This protocol can also be used for 3D morphometric analysis of other cells from either *in vivo* or *in vitro* conditions.

Video Link

The video component of this article can be found at http://www.jove.com/video/53113/

Introduction

In healthy central nervous system (CNS), astrocytes play an important role in the regulation of blood flow, energy metabolism, synaptic function and plasticity, and extracellular ion and neurotransmitter homeostasis[1-3]. In addition, astrocytes respond to different harmful stimuli and abnormal conditions such as trauma, infection, ischemia or neurodegeneration via reactive astrogliosis which is characterized by hypertrophy, proliferation and functional remodeling of astrocytes[4,5].

Reactive astrogliosis can engineer the inflammatory response and repair process in the tissue and, therefore, can affect the clinical outcome of therapeutic interventions. Accordingly, astrocytes have received attention from neuroscientist during the last decades as potential targets for therapeutic interventions for a variety of diseases affecting the CNS.

Astrocytes normally have a stellate shape with well-defined branches that spread around the soma[6]. In a diseased condition in the brain, astrocyte branches become convoluted and show swollen ends[7], for example in the presence of amyloid beta (Aβ).

This article presents a protocol for analyzing 3D images of astrocytes acquired by confocal microscopy. Twelve different quantitative parameters for each astrocyte were measured: the surface areas and volumes of the astrocyte territory (the tissue covered by an astrocyte), entire cell (including branches), cell body, and nucleus; the total length and number of branches; the fluorescence intensity of antibodies used for astrocyte detection; and the density of astrocytes (number/1,000 μm^2). For this purpose, we used brain sections from rats exposed to intrahippocampal injection of $A\beta_{1-40}$ with or without genistein treatment as an anti-inflammatory substance. The described protocol can be used for morphometric analysis of different cell types *in vitro* or *in vivo* in different conditions.

Protocol

This study was carried out in accordance with the policies set forth in the Guide for the Care and Use of Laboratory Animals (NIH) approved by Ethic Committee of Iran University of Medical Sciences (Tehran, Iran).

1. Animals, Surgery and Specimen Preparations

NOTE: Prepare brain tissue for 3D confocal microscopic analysis.

1. Divide animals randomly into two groups: $A\beta_{1-40}$–injection (n = 8), and $A\beta_{1-40}$–injection with genistein treatment (n = 8); administer genistein (10 mg/kg diluted in a vehicle such as polyethoxylated castor oil) by gavage 1 hr before surgery.
2. Anesthetize the animals with ketamine (100 mg/kg) and xylazine (10 mg/kg). Confirm proper anesthesia by lack of withdrawal reflex after pinching the toe. Use ointment on eyes during the surgery to prevent dryness.
3. Place animals in stereotaxic apparatus and shave head. Apply iodine solution to clean the scalp before incision and keep the operation site sterile during the surgery to reduce the risk of infection. Inject $A\beta_{1-40}$ stereotaxically (4 µl) in the hippocampus at −3.5 mm posterior to bregma, ± 2 mm lateral to midline, and −2.8 mm below dura, according to rat brain atlas[8]. For stereotaxic method please see previous publication[9].
4. Provide post-operative observation/care until the animals are fully conscious to ensure the comfort of the animals. Keep the animals warm during recovery, and do not return them to a cage with other animals until full recovery. Pay attention to any sign of infection in the animals after surgery.
5. Anesthetize the animals deeply by ketamine (150 mg/kg) three weeks after surgery. Perform transcardial fixation by perfusing the animals with 0.9% saline followed by 4% paraformaldehyde in 0.1 M PBS (pH = 7.4), then remove and fix brain as described in[10].
6. Post-fix the brains in 4% paraformaldehyde for 2-3 days at 4 ˚C.
7. Embed the tissue in paraffin blocks by use of tissue embedding equipment, and avoid under-filling or over-filling the cassette since it may interfere with correct alignment or sectioning. Pay attention to the orientation of the tissue in the paraffin block.
   NOTE: The rat hippocampus, for example, is close to posterior part of the cerebral hemisphere. Thus, the tissue should be embedded in a way that sectioning starts at posterior part of the brain. Use the Paxinos atlas[8] as a guide to reach the desired anatomical structure.
8. Place the microtome away from air drafts or doorways since air movements make the handling of sections difficult.
9. Use sections with a thickness ≥20 µm as this depth with be necessary to produce Z-Stack images of complete astrocytes. Do not use the first couple sections, as they may have undesired thickness due to thermal expansion.
10. Place the sections on the surface of warm water (5 ± 2 ˚C, below the melting temperature for paraffin) just long enough to flatten the sections.
    NOTE: Over expansion of the section can disturb the morphology of the structure. Use tiny paintbrush to carefully transfer sections. Collect two to three sections on each slide.
11. Store slides in a rack in an upright position and let them dry at 37 ˚C for several hr or O/N.

2. Immunohistochemistry

1. Deparaffinize sections in xylene, 2 x 10 min, and rehydrate through alcohol gradient (99.8%, 95%, and 70%, 10 min each) and PBS.
2. Incubate with antibodies against glial fibrillary acidic protein (GFAP) diluted 1:1,500 in PBS containing 0.25% bovine serum albumin (BSA), 0.25% Triton X-100 and 3.5% normal serum (O/N at 4 ˚C).
3. Wash sections in PBS for 3 x 5 min.
4. Incubate with secondary alkaline phosphate-conjugated antibodies for 1h (1:100, 21 ˚C, diluted in PBS).
5. Wash the sections in PBS for 3 x 5 min.
   NOTE: It is important to wash the sections properly after secondary antibodies to minimize background staining.
6. Incubate sections in darkness with Liquid Permanent Red Chromogen (15-20 min). Note: Prepare the solution no more than 30 min before use.
7. Wash sections in PBS for 3 x 5 min.
8. Finally, stain the nuclei with 4-6-diamidino-2-phenylindole (DAPI; 1:500, diluted in PBS) prior to cover-slipping. Store the slides in the refrigerator 24-48 hr before microscopy.

3. Confocal Microscopy

NOTE: For quantitative evaluation (see below), select an astrocyte with a clearly visible DAPI-stained nucleus with a minimum of overlapping branches to reduce the risk of errors. Producing Z-Stack images is time consuming. Be patient and do not stop during processing. Upright confocal laser scanning microscope is used to create 3D images from astrocytes.

1. Put the slide on the microscope-stage, and select the 63X objective.
2. Open the imaging software and click on Start system. Click on the Locate tab. Go to Assign, and select GFP to adjust the proper light.
3. Open the Shutter to reflect light. Find the Reflector Revolver on the right side of Shutter and choose the color that should be reflected by astrocytes (green, red or violet); red color (FSet20 wf) was used here.
   NOTE: Do not forget to focus the picture directly in the ocular of the microscope.
4. To find the best focus, click on All closed, put the microscope pin in screen mode, and click on the Acquisition tab.
5. Click on Smart Setup under the Acquisition tab; a window opens. Select the proper fluorophore (*i.e.* DAPI and Alexa Fluor 555, in the current project) from the list. The color is automatically selected.
6. Click on Best signal, and Apply, then click on Set Exposure; the computer will then automatically set the exposure parameters.
7. For optimizing the image, go to the Channels window. Unmark Track2, highlight Track1 and click on Live. Focus the image if needed.
8. In the Channels window adjust the following keys; click on 1AU to automatically optimize the pinhole. It is very important to do this step otherwise the confocal images will be non-optimal. Adjust Gain to have the best intensity (keep this setting below 800 to reduce extra noise). Finally, set the Digital Gain between 2 and 3.
9. Unmark Track1, click on Track2 and repeat Step 3.8 for Track2. Then stop Live.
10. Go to the Acquisition Mode window. Improve the image by optimizing Frame Size and Averaging. In order to change frame size go to X*Y and select 1,024 x 1,024. Choose a slow Speed to create a better image.
11. Go to Averaging and select a Number ≥4. This averaging value indicates the number of scans that will be averaged to produce the acquired image. Now click on the Snap button, and save the captured 2D image.

12. For 3D imaging, mark the Z-Stack item under Smart Setup causing the Z-Stack window to open.
13. Set the first and last positions for the Z-Stack, *i.e.* the depth of the cell. First click on Live. Then use the focus drive of the microscope to focus on the uppermost position of astrocyte in the tissue, where the Z-Stack is to start. Click on Set First. Then focus down to the lowest position of the astrocyte in the tissue, where Z-Stack scanning should be stopped. Click on Set Last. Then stop Live.
14. Choose the Interval; 1.01 µm is used in the current study; choosing interval can be done by clicking on Optimal to set the number of slices.
15. Click on Start Experiment. Save the images once the scan is complete. This image will be used for the measurement of the following parameters: surface area and volume of astrocyte territory, the entire astrocyte including branches, cell body, and nucleus.
16. Acquire a 2D image using a 10X or 20X objective as described in Steps 3.6-3.11. This image can be used for the measurement the intensity of fluorescence immunoreactivity of antibodies, and astrocyte density (number/1,000 μm^2).

4. Astrocyte Density and GFAP$^+$ Fluorescence Intensity

1. Open the 20X 2D image. Click on Histo (Hisogram) on the left side of image window.
2. To calculate astrocyte density, select three consecutive visual fields under 20X objective. Count the number of astrocytes that exhibit a clear soma with minimum, overlapping branches in the fields.
3. To quantify fluorescence intensity, select a proper tool (rectangle or circle) on the footer of image window, and choose an area for the measurement. Note the mean value and standard deviation appear automatically under the image. In the current study, a 0.7 mm^2 rectangular area between striatum and medial blade of dentate gyrus was used.

5. Astrocyte Branches

1. To quantify the length and total number of branches, use 3D images (acquired with 20X) in the image analyzing software. Draw a line manually along the length of each branch of the selected astrocyte. Then select the measurement-tool that is specialized for measuring line-length. In order to quantify the number of branches, select the count-tool to count the number of marked items.

6. Volume and Surface Area

NOTE: Marking a structure manually in 3D image analysis software requires focus and training. Practice several times before starting an experiment.

1. To quantify the volume and surface area of the astrocyte nucleus, soma, cell body and territory, transfer the 3D images (acquired in Steps 3.1-3.16) to a 3D image analysis software such as Volocity 6.1.
2. Open the software and create a new library. Drag all images to the left gray column in the new library. Then, select one 3D image. On the right, there are different channels with different colors. Turn on one channel of interest; for example the blue channel for DAPI when measuring the nucleus. Change the brightness manually to get an optimal contrast.
3. Select the Tools menu, go to Make Volume and produce a single 3D image out of Z-stack images. Click on Properties in the Edit menu and be sure that X, Y and Z properties are presented; otherwise it is impossible to carry out 3D analysis.
4. Click on Freehand RIO tool icon in the toolbar. To measure the volume and surface area of astrocyte nucleus draw a line around the DAPI–labeled nucleus.
5. To measure the volume and surface area of astrocyte cell body draw a line around the soma excluding branches.
6. To measure the volume and surface area of entire astrocyte (cell body including branches) draw a line around the soma following the surface of all branches.
7. To assess the volume and surface area of astrocyte territory draw a line between tips of the branches.
   NOTE: Press Delete on the keyboard to erase unwanted drawings.
8. Click on the Measurements menu at the top of the image window to create a measurement protocol; a window containing different tools opens.
9. Drag Find objects to top of the window (pane). Give a descriptive name to the Population 1, select the associated color, and click on Measure. Another window opens.
10. Choose a parameter *i.e.*, volume or surface area. Click on OK. The data will appear in a table.
11. To save the data click on the Measurement menu and select Make Measurement Item.
12. Select A new measurement item called from the opened window. Then give a name to the data and click on OK.
13. Finally, export the data to statistics software such as Graph pad or excel program. Use t-test to analyze the data, then plot 2D graphs.

Representative Results

This section presents some examples of the qualitative and quantitative observations produced by 3D morphometric analysis. For complete results of all 12 parameters mentioned earlier, please see our previous publication[10].

Qualitative Observations

Astrocytes exhibited thin or thick branches that were usually long in $A\beta_{1-40}$ injected rats (**Figure 1**). A few small stellate-shaped astrocytes with short branches were observed in the cerebral cortex, and a compact network of astrocytes occurred in the corpus callosum.

Astrocytes in the brain tissues of animals with $A\beta_{1-40}$–injection followed by genistein treatment exhibited a stellate form with short and thin branches (**Figure 2**)[10]. A few astrocytes showed an atrophic appearance.

Astrocyte Density and GFAP$^+$ Fluorescence Intensity

The mean number of astrocytes/1,000 μm^2 was significantly higher in rats with $A\beta_{1-40}$–injection in comparison with animals in $A\beta_{1-40}$–injection and genistein treatment group (P <0.0001). In addition, the $GFAP^+$ fluorescence intensity was significantly lower in the brain section with genistein treatment compared with non-treated $A\beta_{1-40}$ injected animals (**Figure 3**).

Morphometric Analysis of Astrocytes Volume

The volume of the astrocyte nucleus, cell body, entire astrocyte and astrocyte territory decreased significantly in the genistein treatment group compared with untreated animals. This result suggests that genistein ameliorated the astrogliosis caused by the presence of amyloid (**Figure 4A**-**D**).

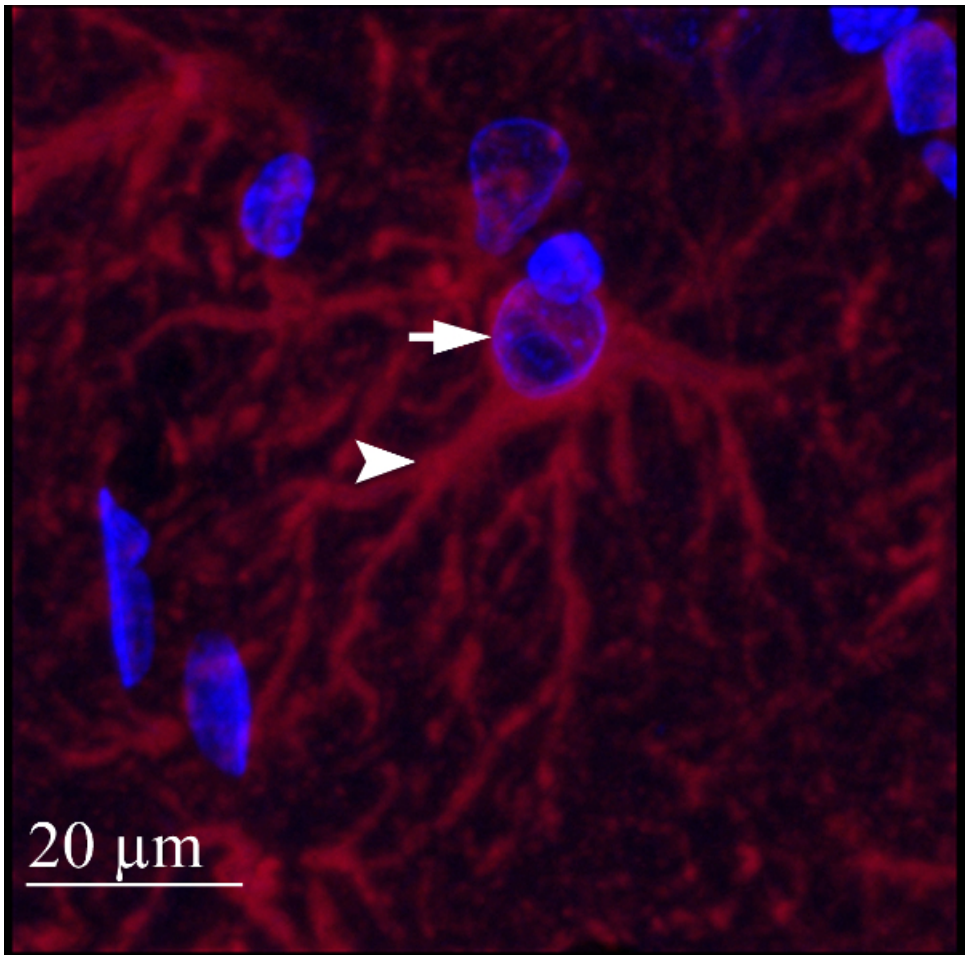

Figure 1. Confocal image of an astrocyte. An individual astrocyte with thick and long branches (arrowhead) and DAPI-labeled nucleus (arrow) in a rat brain subjected to hippocampal injection of $A\beta_{1-40}$. Please click here to view a larger version of this figure.

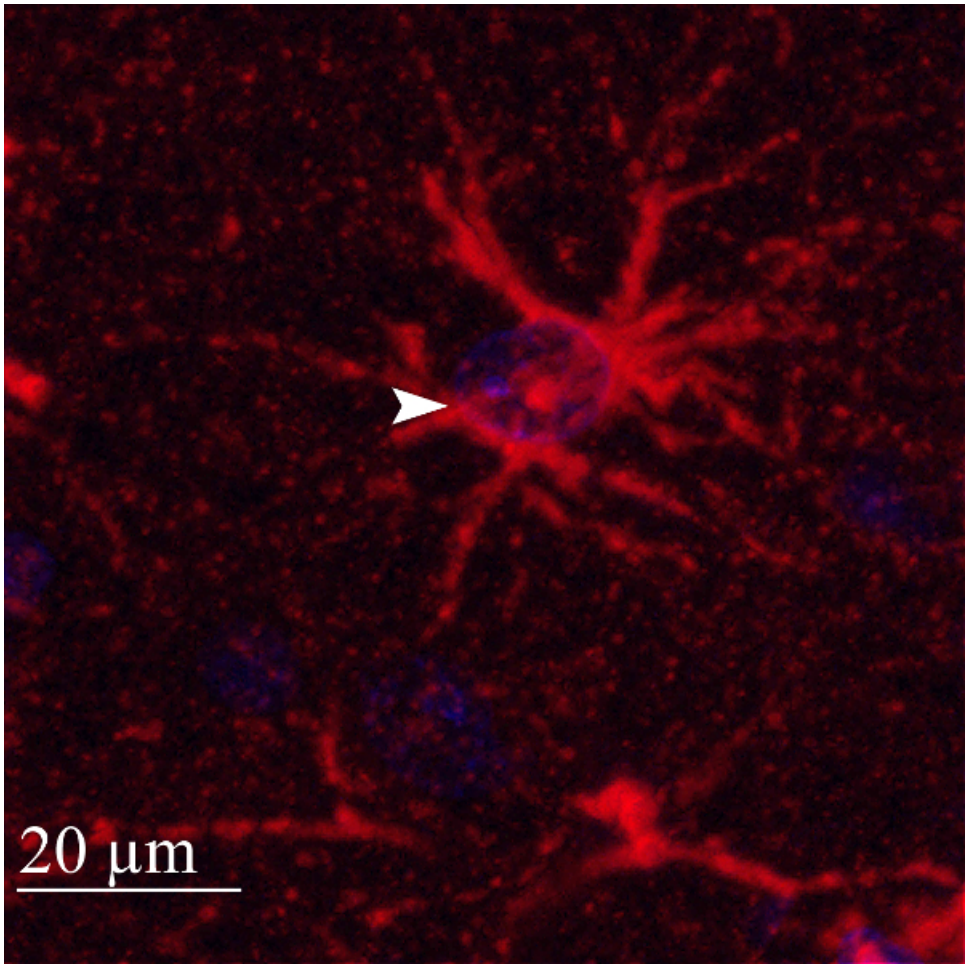

Figure 2. Confocal image of an astrocyte. An astrocyte with short branches (arrowhead). In a rat brain subjected to hippocampal $A\beta_{1-40}$–injection, and genistein pre-treatment administered by gavage. Please click here to view a larger version of this figure.

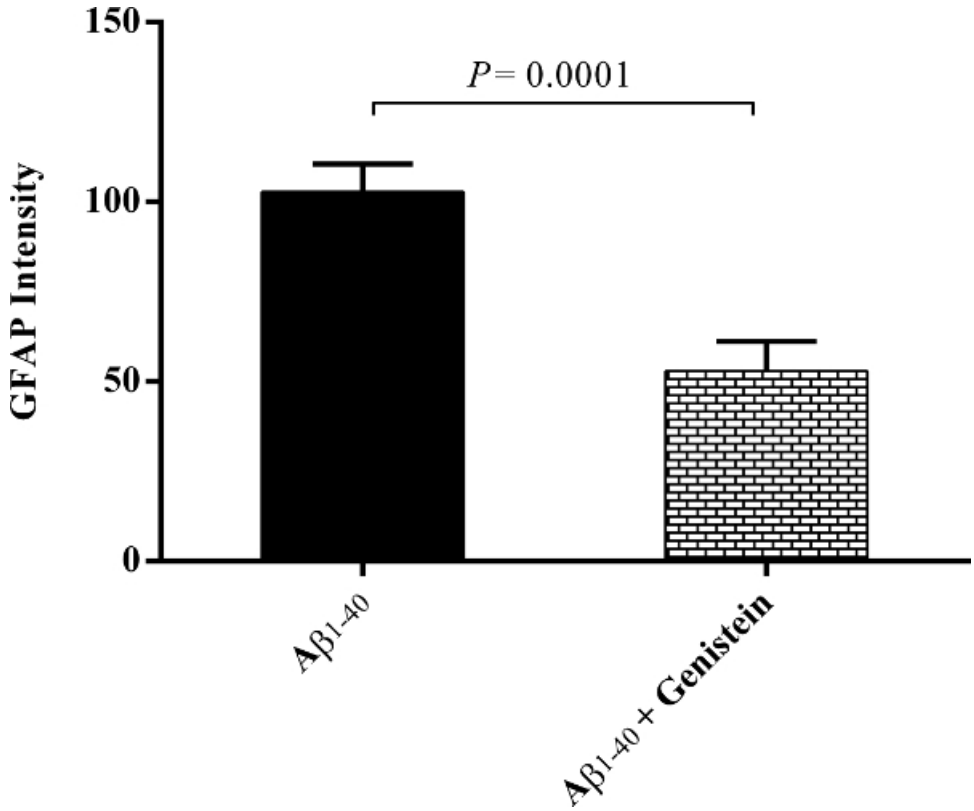

Figure 3. GFAP$^+$ fluorescence intensity decreased significantly in animals with $A\beta_{1-40}$–injection that received genistein pre-treatment. Values are mean ± SEM. Fifty astrocytes per animal were evaluated. *P* <0.05 was regarded as significant, and T-test was used to compare the data between groups with or without treatment. Please click here to view a larger version of this figure.

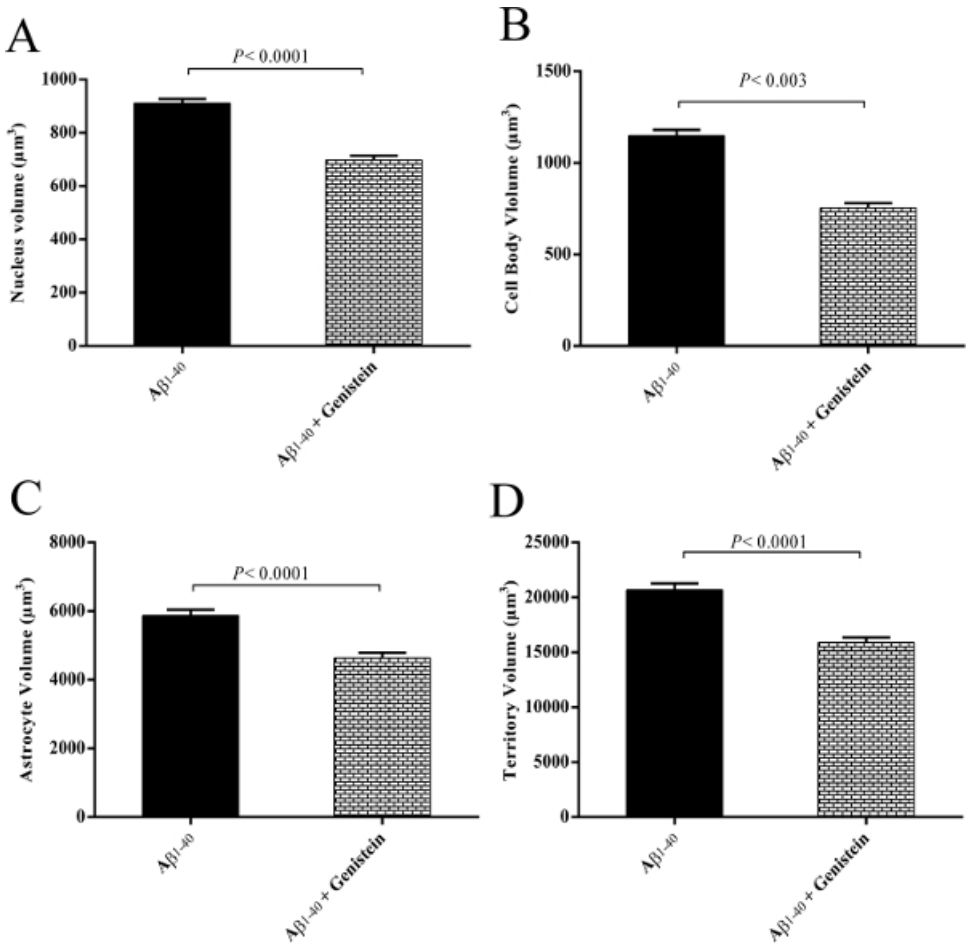

Figure 4. The mean volume of nucleus (A), cell body (B), astrocyte (including branches; (C), and territory (D) in brain samples taken from rats subjected to $A\beta_{1-40}$–injection, with or without genistein pre-treatment. Genistein significantly ameliorated the enlargement of nucleus, cell body, the entire astrocyte, and astrocyte territory (see ref[10]). Values are mean ± SEM. Fifty astrocytes per animal were evaluated. *P* <0.05 was regarded as significant, and T-test was used to compare the data between groups before and after treatment. Please click here to view a larger version of this figure.

Discussion

In the current protocol, we employed 3D confocal morphometry to evaluate 12 different parameters that were associated with astrocyte morphology. For this purpose, hippocampal tissue of rats with $A\beta_{1-40}$–induced astrogliosis, with or without genistein pretreatment as an anti-inflammatory agent were used. By using 3D images and morphometric software, we were able to show the effect of genistein on astrogliosis *i.e.* the morphology of astrocytes.

Changes in the intra- and extra cellular environment of the brain tissue can alter volume and/or size of cells/tissue. These alterations are considered pathological hallmarks in various brain injuries[3,11]. In order to quantify these alterations, a number of techniques are used. Most investigations are based on using 2D low resolution images captured by light- or electron microscopy (EM) that give information only about a small fraction of the cell. To overcome this limitation, 3D confocal morphometry on high resolution images was developed[11]. Another advantage of confocal microscopy is that the architecture of a cell can be studied since several consecutive 2D images are obtained while Z-Stack imaging is performed. In addition, 3D confocal microscopy raises the possibility to improve the quality of the images by filtering background noise[11].

The method presented here is time-consuming; taking Z-Stack images with a confocal microscope takes several minutes for each single cell. In addition, manually marking a structure in software requires some practice, and the researcher may need to redo the drawing several times to be sure that the structure is correctly marked. It should be mentioned that the 'Magic' tab in 3D image analysis software such as Volocity, can be used to automatically mark all DAPI–stained nuclei in a single image. This option can be used when astrocytes are cultured. The brain sections, however, contain not only astrocytes but also many other cells such as microglia and neurons. This means that many of the DAPI-stained nuclei do not belong to astrocytes. The nuclei associated with astrocytes should be manually marked using images having both DAPI-labeled nuclei and GFAP-immunoreactive astrocytes.

In this protocol, antibodies against GFAP were used to visualize astrocytes in paraffin sections. Astrocytes can also be visualized by using other antigens[12,13], or transgenic mice. The investigators should be aware of limitations of the methods, and use the best possible experimental design based on their aims. The limitation of using antibodies against GFAP, for example, is that only 10-15% of the cytoskeleton can be detected. This limitation, however, can become an advantage when studying astrogliosis; the increase number of astrocytes and a dense network of

astrocytes' branches in astrogliosis can make it difficult to identify a single cell if a larger area of the cell is detected. Although this protocol describe the quantification of 12 different parameters but the investigator can choose the ones that suit their aim. According to our experience, the measurements of all parameters can give valuable information about the parameters that are significantly affected in a certain condition or by a treatment. The quantification method performed on 3D pictures captured in confocal microscope can also be performed on frozen sections. The issue is to treat the samples (fixation, dehydration, embedding and sectioning) in a similar way since these steps can lead to shrinkage or swelling of the tissue.

In conclusion, 3D confocal imaging, in combination with morphometric software, makes it possible to quantify several parameters associated with the morphology of a cell. The protocol we presented here can be used for evaluation of morphological changes that appear in a cell, in different pathological conditions, or as the effect of an intervention strategy.

Disclosures

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